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PRINCIPAL INVESTIGATOR: David J. Riese II, Ph.D.
David F. Stern, Ph.D.

CONTRACTING ORGANIZATION: Yale University School of Medicine
New Haven, Connecticut 06520-8047

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6. AUTHOR(S)
David J. Riese II, Ph.D.
David F. Stern, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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New Haven, Connecticut 06520-8047

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We are studying how the signaling network composed of the Epidermal Growth Factor (EGF) family of peptide hormones and the ErbB family of receptor tyrosine kinases is differentially activated and coupled to physiologic responses. Because deregulation of this signaling network plays a significant role in the genesis or progression of several different human metastatic diseases, an understanding of the basic "wiring" of this network is required to elucidate the mechanisms of these diseases. We have generated a panel of cell lines based on the Interleukin-3 (IL3) -dependent Ba/F3 mouse hematopoietic cell line that ectopically express the four erbB family receptors. We have then identified the patterns of ErbB family receptor phosphorylation and IL3-independent responses stimulated by different EGF family hormones. We will present data supporting several concepts that have emerged from these studies:

(1) Differential Activation by EGF Family Hormones. EGF family hormones can be assigned to three distinct functional groups based on their stimulation of ErbB family receptor phosphorylation.

(2) Differential Coupling by ErbB Family Receptors. ErbB family receptors and combinations of receptors differentially couple to IL3-independent responses in Ba/F3 cells. Furthermore, ErbB family receptors directly activated by ligand binding behave differently from receptors activated in trans through receptor heterodimerization.

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Progress Report

III. Body - Assessment of ★Proposal Technical Objectives and ✣Other Experiments

I. Introduction

A. Introduction to the problem

Breast cancer is one of the leading causes of cancer death in women in the United States; approximately 46,000 American women died of the disease in 1993 [Boring, *et al.*, 1993]. One model is that breast cancer results from disruptions in the normal hormonal regulation of mammary gland epithelial cell proliferation and differentiation. We have undertaken experiments to characterize the signaling network composed of the epidermal growth factor (EGF) family of peptide hormones and the ErbB family of receptor protein tyrosine kinases. We also have begun experiments that assess the effects of this network on the proliferation, differentiation, and malignant growth transformation of mammary epithelial cells. Therefore, these experiments are beginning to shed light on the role that these proteins play in breast carcinogenesis.

B. The EGF family/erbB receptor family signaling network

Deregulated signaling by the four receptor tyrosine kinases encoded by the ErbB gene family (ErbB1/epidermal growth factor receptor [EGFR], Neu/ErbB2/HER2 , ErbB3/HER3 , and ErbB4/HER4), has been implicated in a number of human cancers, including mammary cancer, ovarian cancer, gastric cancer, and glioblastoma [Reviewed in Hynes and Stern, 1994]. Understanding the normal and pathological functions of these receptors requires that their regulation by hormones be elucidated. However, there are at least 9 different agonists for ErbB family receptors, including epidermal growth factor (EGF), transforming growth factor alpha (TGFα), amphiregulin (AR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), cripto (CR), epiregulin (EPR), the neuregulins (NRGs - also known as gp30, heregulins, neu differentiation factors, glial growth factors, and

acetylcholine receptor inducing activity), and the neuregulin2s (NRG2s) [Reviewed in Groenen, *et al.*, 1994; Chang, *et al.*, 1997; Carraway, *et al.*, 1997]. While a single ErbB family receptor can in some cases bind more than one ligand (e.g. EGFR) [Riese, *et al.*, 1996b], a single EGF family hormone can in some cases bind to multiple ErbB family receptors (e.g. BTC, EPR) [Riese, *et al.*, 1996a; Riese, *et al.*, submitted]. Furthermore, while an ErbB family receptor expressed by itself may not be stimulated by a given EGF family hormone, this receptor can be activated when co-expressed with another ErbB family receptor that can bind the hormone [Reviewed in Earp, *et al.*, 1995]. For example, EGF does not bind or activate ErbB2 expressed on its own, but activates ErbB2 when coexpressed with the EGFR [Akiyama, *et al.*, 1988; King, *et al.*, 1988; Stern and Kamps, 1988; Connelly and Stern, 1990]. This "transmodulation" activation of ErbB2 by EGFR apparently occurs through the formation of EGF-driven receptor heterodimers and receptor cross-phosphorylation [Goldman, *et al.*, 1990; Wada, *et al.*, 1990; Qian, *et al.*, 1992; Spivak-Kroizman, *et al.*, 1992].

The physiological responses to agonists for ErbB family receptors depends on their ability to coordinately activate multiple receptors that are differentially expressed and have different signaling capabilities. Although NRGs were initially purified by their ability to induce ErbB2 tyrosine phosphorylation, and were thought to be ligands for ErbB2, NRG does not bind ErbB2 and/or induce ErbB2 tyrosine phosphorylation in a variety of cell types or in solution [Culouscou, *et al.*, 1993; Peles, *et al.*, 1993; Plowman, *et al.*, 1993b; Sliwkowski, *et al.*, 1994; Tzahar, *et al.*, 1994]. Instead, NRG binds ErbB3 [Carraway, *et al.*, 1994; Kita, *et al.*, 1994; Sliwkowski, *et al.*, 1994; Tzahar, *et al.*, 1994] and ErbB4 [Plowman, *et al.*, 1993a; Plowman, *et al.*, 1993b; Culouscou, *et al.*, 1993]. Co-expression of ErbB3 or ErbB4 with ErbB2 permits NRG-induced tyrosine phosphorylation of ErbB2, presumably through the formation of

Neu/ErbB3 or Neu/ErbB-4 heterodimers [Plowman, *et al.*, 1993b; Carraway, *et al.*, 1994; Kita, *et al.*, 1994; Sliwkowski, *et al.*, 1994; Riese, *et al.*, 1995]. Despite the many combinatorial possibilities afforded by assortment of four different receptors, interactions of ErbB family members with their agonists have only been investigated in a piecemeal fashion. Receptors of different species origins have been mixed in gene transfer experiments, only a subset of receptor combinations have been tested, and interpretation is hampered by the variety of cell backgrounds used and in many cases by the failure to determine the endogenous ErbB family receptor expression in the cell lines used. Finally, the hormone-regulated coupling of different ErbB family receptors and combinations of receptors to different downstream signaling pathways has not been systematically investigated for any EGF family agonist.

C. Experimental approach

In order to address these issues we have undertaken a parallel analysis of the aggregate signaling potential of the ErbB receptor family. We have expressed all four human ErbB family receptors, singly and in each pairwise combination, in the interleukin-3-dependent mouse Ba/F3 pro-B-lymphocyte cell line. By assessing ErbB receptor tyrosine phosphorylation in the resulting panel of cell lines following stimulation with every available EGF family hormone, we have performed the first comprehensive evaluation of ligand-induced ErbB family receptor activation. Furthermore, by assessing the induction of IL-3-independent growth in this panel of cell lines following hormonal stimulation, we have also evaluated ligand-induced erbB family receptor coupling to cellular signaling pathways. These data establish that different EGF family hormones stimulate distinct patterns of ErbB receptor phosphorylation and coupling to cellular signaling pathways. Moreover, these data

suggest several additional mechanisms by which biological responses are specified by interactions among ErbB family receptors and their agonists.

II. Materials and Methods

A. EGF Family Hormones

The production and/or synthesis of recombinant AR, NRGβ, and BTC have been described [Riese, *et al.*, 1995; Riese, *et al.*, 1996a; Riese, *et al.*, 1996b]. Recombinant EGF and TGFα were obtained from Collaborative Biomedical. Recombinant AR, BTC and NRGα were obtained from R&D Systems. Recombinant HB-EGF has been provided on a collaborative basis by Michael Klagsbrun (Children's Hospital, Boston, MA). Recombinant EPR has been provided on a collaborative basis by Taisho Pharmaceuticals (Tokyo, Japan). CR peptide has been provided on a collaborative basis by William J. Gullick (ICRF) and David S. Salomon (NCI/NIH). Recombinant NRG2 has been provided on a collaborative basis by Han Chang (Stanford University).

B. Cell lines and cell culture.

The Ba/F3 mouse pro-B-lymphocyte cell line [Palacios and Steinmetz, 1985] and its derivatives were grown in RPMI (Gibco/BRL) supplemented with 10% fetal calf serum (Sigma) and Interleukin-3 (IL-3) supplied as 10% conditioned medium from the WEHI-3B mouse myelomonocytic leukemia cell line [Daley and Baltimore, 1988]. Ba/F3 derivatives transformed with constructs expressing ErbB family receptors were grown in medium supplemented with 200ug/ml G418 (Gibco/BRL).

C. Plasmid constructions and generation of recombinant Ba/F3 derivatives.

The construction of recombinant retroviral vectors expressing the four ErbB family receptor cDNAs have been described previously [Riese, *et. al.*, 1995]. The generation and characterization of Ba/F3 derivatives expressing the four ErbB family receptors, singly and in every pairwise combination, have also been described previously [Riese, *et al.*, 1995].

D. Stimulation and analysis of erbB family receptor tyrosine phosphorylation.

The stimulation and analysis of ErbB family receptor tyrosine phosphorylation have been described earlier [Riese, *et al.*, 1995].

E. Stimulation and analysis of IL-3 independent responses.

The stimulation and analysis of IL-3 independent responses have been described earlier [Riese, *et al.*, 1995].

III. Body - Assessment of Proposal Technical Objectives and Other Experiments

A. Subclone erbB receptor and neuregulin cDNAs into retrovirus-based expression vectors and generate recombinant retroviral stocks to facilitate gene transfer and expression.

We have subcloned the four different erbB family receptor cDNAs into the pLXSN recombinant retrovirus-based expression vector [Riese, *et al.*, 1995]. We have also subcloned the neuregulin-α cDNA into this same vector [Riese and Stern, unpublished data]. While we have packaged these constructs into recombinant retroviral stocks, these stocks were not used to generate the Ba/F3 derivatives described elsewhere in this report.

B. Develop cultured cell systems for expression and purification of recombinant neuregulin.

We have introduced a neuregulin-α (NRGα) expression vector into mouse C127 fibroblasts, generating cell lines that stably express and secrete NRGα. We have also engineered recombinant baculovirus stocks containing a NRGα cDNA. However, we have not conclusively determined that infection of insect cells with these stocks results in the production of biologically active NRGα. Dr. Frank Jones, a postdoctoral fellow in the Stern Laboratory, is continuing these efforts to produce biologically active recombinant NRG.

To bypass these difficulties in producing recombinant NRG, we established a collaboration with Drs. James D. Moyer, Brad C. Guarino, and Glenn C. Andrews, Pfizer Central Research, Groton, CT. They have supplied us with NRGβ as a refolded, biologically active, chemically-synthesized 65-mer peptide corresponding to amino acids 177 to 241 of the NRG β1 isoform [Riese, *et al.*, 1995; Barbacci, *et al.*, 1995]. We also established a collaboration with Drs. Sharon Buckley and Gregory D.

Plowman, Sugen, Inc., Redwood City, CA. They have supplied us with human recombinant betacellulin (BTC) and human recombinant amphiregulin (AR) as refolded, biologically active peptides expressed in *E. coli.* We also established collaborations with Dr. Michael Klagsbrun (Children's Hospital, Boston, MA), who have supplied us with recombinant heparin-binding EGF-like growth factor (HB-EGF), with Dr. David Salomon (NIH/NCI) and Dr. William J. Gullick (ICRF), who has supplied us with cripto peptide (CR), with Dr. Han Chang (Stanford University), who has supplied us with recombinant Neuregulin2 (NRG2), and with Taisho Pharmaceuticals, Tokyo, who has supplied us with recombinant epiregulin (EPR). We have purchased recombinant transforming growth factor alpha (TGFα) and EGF from Collaborative Biomedical, and recombinant AR, NRGα, and BTC from R&D Systems.

C. Determine what erbB receptors are neuregulin effectors in Ba/F3 cells

As described previously, neuregulin-β (NRGβ) stimulates ErbB4 tyrosine phosphorylation as well as ErbB3 tyrosine phosphorylation, but activates ErbB3 only when ErbB3 is coexpressed with another ErbB family receptor. Furthermore, NRGβ stimulates EGFR and ErbB2 tyrosine phosphorylation when these receptors are coexpressed with either ErbB3 or ErbB4 [Riese, *et al.*, 1995] (Figure 1).

D. Determine if neuregulin acts as an adhesion molecule or receptor for erbB proteins.

These experiments are being performed by Jonathan McMenamin-Balano, a predoctoral student in the Stern Laboratory. Preliminary evidence suggests that the membrane-bound, immature form of neuregulin acts as a receptor for ErbB3 or ErbB4 and that the cytoplasmic domain of neuregulin is coupled to cellular signaling pathways.

E. Assess effector-specific or presentation specific neuregulin-induced protein tyrosine phosphorylation.

These experiments are being performed by Jonathan McMenamin-Balano, a predoctoral student in the Stern Laboratory. Preliminary evidence suggests that soluble and membrane bound forms of neuregulin stimulate identical patterns of erbB family receptor tyrosine phosphorylation.

F. Identify the patterns of erbB receptor tyrosine phosphorylation stimulated by other EGF family hormones.

As summarized in Figure 1, EGF, AR, TGFα, HB-EGF, and EPR stimulate EGFR tyrosine phosphorylation, as well as the phosphorylation of any other ErbB family receptor when it is coexpressed with the EGFR [Riese, *et al.*, 1996a; Riese, *et al.*, 1996b; Riese, *et al.*, submitted]. Surprisingly, BTC and EPR stimulate both EGFR and ErbB4 tyrosine phosphorylation. Furthermore, BTC stimulates both ErbB2 and ErbB3 tyrosine phosphorylation when these receptors are coexpressed with the EGFR. In contrast, BTC stimulates ErbB2 but not ErbB3 tyrosine phosphorylation when these receptors are coexpressed with ErbB4 [Riese, *et al.*, 1996]. Therefore, NRG-β, EGF, and BTC all stimulate distinct patterns of ErbB family receptor tyrosine phosphorylation, which may in part account for their differential biological activities.

G. Determine if hormone-induced erbB receptor signaling confers IL-3 independent growth to Ba/F3 cells.

While NRG-β, BTC, and EGF can all stimulate the tyrosine phosphorylation of all four ErbB family receptors, either directly or through transmodulation (see previous section), activation of different receptors or combinations of receptors may specify unique biological responses through coupling of each receptor to distinct

cellular signaling pathways. We investigated this possibility by determining whether ligand stimulation enabled survival or growth of the various Ba/F3 derivatives independent of interleukin-3 (IL-3). Ectopic expression of a number of receptors in Ba/F3 cells permits receptor regulation by the cognate ligands, which in some cases relieves dependence on IL-3 for survival or growth: expression of the erythropoietin receptor with Friend Spleen Focus-Forming Virus gp55 permits IL-3-independent proliferation [Li, *et al.*, 1990]. Similarly, EGF stimulation of Ba/F3 cells expressing exogenous EGFR results in EGFR tyrosine phosphorylation and increased cellular DNA synthesis [Collins, *et al.*, 1988; Shibuya, *et al.*, 1990], while stimulation of Ba/F3 derivatives expressing exogenous platelet derived growth factor (PDGF) receptor with PDGF results in receptor tyrosine phosphorylation and IL-3-independent proliferation [Satoh, *et al.*, 1993].

In the absence of ligand, all of the Ba/F3 derivatives remained dependent on IL-3 for survival, even those lines that display substantial basal receptor tyrosine phosphorylation [Riese, *et al.*, 1995]. Activation of either EGFR or ErbB2 in the single recombinant cell lines was associated with IL-3 independent survival but not proliferation, while activation of ErbB3 or ErbB4 in the single recombinants had no biological effect [Riese, *et al.*, 1995; Riese, *et al.*, 1996]. Therefore, ligand stimulation of ErbB phosphorylation was necessary, but not sufficient, for an IL-3 independent response (Table 1).

We also assessed ligand activity in the double recombinant Ba/F3 cell lines. As expected from the responses of the single recombinant cell lines, receptor activation in cells expressing EGFR or ErbB2 conferred, with one notable exception, a minimal response of IL-3 independent survival. For example, in EGFR + ErbB2 (1+2), EGFR + ErbB4 (1+4), ErbB2 + ErbB3 (2+3), and ErbB2 + ErbB4 (2+4) cell lines, receptor activation stimulated a minimum of IL-3-independent survival, while in

ErbB-3 + ErbB4 (3+4) cells none of the ligands stimulated an IL-3 independent response [Riese, *et al.*, 1995; Riese, *et al.*, 1996a; Riese, *et al.*, 1996b]. The exception is the response of 1+3 cells to ligand stimulation. As predicted, BTC and EGF stimulated the IL-3 independent survival of 1+3 cells; however, NRG-β failed to stimulate an IL-3 independent response [Riese, *et al.*, 1995; Riese, *et al.*, 1996a].

In some of the double recombinant cell lines ligand stimulation of coupling of multiple receptors to signaling pathways acted in a non-additive manner to stimulate an IL-3 independent response (Table 1). In 1+2 cells BTC and EGF stimulated IL-3 independent proliferation, while in 1+4 cells EGF, BTC and NRG-β stimulated IL-3 independent proliferation [Riese, *et al.*, 1995; Riese, *et al.*, 1996a; Riese *et al.*, 1996b]. Therefore, while activation of either EGFR alone or ErbB2 alone stimulated IL-3 independent survival, activation of EGFR along with either ErbB2 or ErbB4 conferred IL-3 independent proliferation (Table 1).

H. Identify cellular signaling proteins differentially coupled to each erbB family receptor.

We have previously established the four ErbB family receptor couple to different physiologic responses in Ba/F3 cells (Table 1). This suggests that each receptor is coupled to a distinct set of cellular signaling proteins. We have confirmed this hypothesis by identifying cellular signaling proteins that are differentially coupled to the four ErbB family receptors. In Ba/F3 cells, EGFR activation is accompanied by the phosphorylation of c-Cbl and Shc, two known signaling effectors for EGFR (data not shown). In contrast, activated ErbB4 is not accompanied by c-Cbl or Shc phosphorylation (data not shown). This suggests that Shc and c-Cbl are downstream signaling effectors for EGFR and not for ErbB4. In cells expressing both EGFR and erbB-4, BTC stimulate high levels of EGFR, ErbB4, Shc, and c-Cbl phosphorylation. However, Shc and c-Cbl preferentially complex

with EGFR and not with ErbB4, suggesting again that Shc and c-Cbl are signaling effectors for EGFR and not for ErbB4 (Figure 2). Currently we are performing gene transfer experiments with a dominant negative Shc allele and constitutively active Cbl alleles to determine if activation of Shc and/or Cbl are sufficient or are required for the physiological responses of Ba/F3 cells to EGFR activation.

I. Determine if neuregulin-induced ErbB receptor signaling is capable of transforming the growth or altering the differentiative capacity of MCF-10A cells.

We are currently establishing experimental conditions for this experiment. See the section below for additional information about the effects of ErbB family receptor signaling on MCF-10A cells.

J. Characterize the responses of the MCF-10A cell line to ErbB4 signaling.

There is mounting evidence that while activation of either EGFR or neu stimulates mammary cell proliferation and promotes tumorigenesis, increased ErbB4 signaling may inhibit proliferation or tumorigenesis by stimulating differentiation. Ectopic treatment of breast tumor cell lines with NRG inhibits their growth and stimulates milk protein synthesis [Peles, *et al.*, 1992; Wen, *et al.*, 1992]. and NRG promotes the differentiation of breast cells *in vivo* [Krane and Leder, 1996; Jones, *et al.*, 1996]. Furthermore, agonistic anti-ErbB4 antibodies stimulate the differentiation and inhibit the proliferation of human breast tumor cell lines [Chen, *et al.*, 1996]. Moreover, ErbB4 overexpression in human mammary tumor samples correlates with markers for a more favorable prognosis, suggesting that ErbB4 signaling may inhibit tumorigenicity [Bacus, *et al.*, 1996].

We wished to examine the effects of increased ErbB4 signaling on the proliferation of MCF-10A cells. However, in these cells ErbB4 tyrosine

phosphorylation is not stimulated by either NRG [Beerli, *et al.*, 1995] or BTC (data not shown). Therefore, we tried to establish MCF-10A derivatives that ectopically overexpress ErbB4 through infection with a recombinant retrovirus containing the neomycin resistance gene (neo^R) and the human ErbB4 cDNA (see section II.C.). We noted that this retrovirus stock had an unusally low titer in MCF-10A cells and that the rare transformants that arose from infections with the ErbB4 retrovirus did not express higher levels of ErbB4 than the parental MCF-10A cells (data not shown). A more careful analysis demonstrates that the low titer of the ErbB4 retrovirus stock is specific for ErbB4 (Table 2), suggesting that ErbB4 is a growth suppressor in breast cells through coupling to cellular differentiation. Future experiments will examine the activity of ErbB4 mutants lacking cytoplasmic tyrosine residues that serve as sites for autophosphorylation and docking to receptor effector proteins. These experiments will identify signaling effectors required for the growth inhibitory effect of ErbB4 signaling in MCF-10A cells.

IV. Conclusions/Discussion

We have analyzed the hormone-dependent responses conferred upon the Ba/F3 mouse pro-B-lymphocyte cell line by expression of the four erbB family receptors, singly and in pairwise combinations. In the presence of appropriate co-receptors, NRG regulated the tyrosine phosphorylation of all four erbB family receptors. While some of the NRG-induced interactions between erbB family receptors observed had been predicted from previous work, we demonstrate here for the first time that in the presence of ErbB3 or ErbB4, NRG regulates tyrosine phosphorylation of the EGFR, and that the presence of the EGFR, ErbB2, or ErbB4 enables NRG to regulate tyrosine phosphorylation of ErbB3. Furthermore, NRG induces IL-3-independent survival or proliferation in only a subset of the lines that exhibit NRG-induced receptor tyrosine phosphorylation. Thus the biological responses to NRG are specified at several different levels of regulation.

Previous analyses of NRG-induced signaling by ErbB family receptors have been carried out with a few receptor combinations in a variety of cell backgrounds. In mammary cells, NRGs induce ErbB2 tyrosine phosphorylation, can be cross-linked to ErbB2, and binding is increased by ErbB2 overexpression [Peles, *et al.*, 1993]. This suggests that ErbB2 is a receptor for NRG. However, NRG fails to induce ErbB2 tyrosine phosphorylation and/or bind ErbB2 when ErbB2 is expressed in fibroblasts, ovarian cells [Peles, *et al.*, 1993], CHO cells [Culouscou, *et al.*, 1993; Plowman, *et al*, 1993b], T-lymphoid cells [Plowman, *et al.*, 1993b], or COS-7 cells [Sliwokowski, *et al.*, 1994], and NRG does not bind to solubilized ErbB2 extracellular domains [Tzahar, *et al.*, 1994]. Moreover, NRG binds ErbB3 [Carraway, *et al.*, 1994; Kita, *et al.*, 1994; Sliwkowski, *et al.*, 1994; Tzahar, *et al.*, 1994] or ErbB4 [Culouscou, *et al.*, 1993; Plowman, *et al.*, 1993a; Plowman *et al.*, 1993b; Tzahar, *et al.*, 1994], and co-expression

of ErbB3 or ErbB4 with ErbB2 confers NRG responsiveness upon ErbB2, probably through the formation of ErbB2/ErbB3 or ErbB2/ErbB4 heterodimers [Carraway, *et al.*, 1994; Kita, *et al.*, 1994; Plowman *et al.*, 1993b; Sliwkowski, *et al.*, 1994]. This has led to the general working hypothesis that activation of ErbB2 by NRG requires the presence of ErbB3 or ErbB4.

The present data are compatible with this conclusion, and extend the model to include NRG regulation of the EGFR. The EGFR and ErbB3 alone fail to respond to NRG for two different reasons. The EGFR does not bind NRG [Holmes, *et al.*, 1992], whereas ErbB3 binds, but is impaired for kinase activity [Guy, *et al.*, 1994]. The stimulation of tyrosine phosphorylation of ErbB2 by NRG might suggest direct activation of ErbB2 by NRG, but in view of previously published work, is more likely to reflect interaction with endogenous ErbB3. However, ErbB4 is able to bind and respond to NRG directly.

NRG induces extensive cross-talk among receptors expressed in binary combinations. Either ErbB3 or ErbB4, both of which bind NRG, enable regulation of the EGFR by NRG. This is the first evidence that NRG can regulate EGFR signaling. As predicted from earlier work, NRG stimulates tyrosine phosphorylation of both receptors in the 2+3, 2+4, and 3+4 cell lines. Coexpression of EGFR, ErbB2, or ErbB4 with ErbB3 permits NRG induction of ErbB3 tyrosine phosphorylation. Although earlier work showed that expression of neu enhances tyrosine phosphorylation and NRG regulation of ErbB3 [Carraway, *et al.*, 1994], that work was done in COS-7 cells, which express significant basal amounts of ErbB2 and EGFR. Thus the present work demonstrates for the first time that *de novo* expression of either the EGFR, ErbB2, or of ErbB4 enables hormone-regulated phosphorylation of ErbB3. Endogenous ErbB family receptor expression in Ba/F3 cells played a limited, yet significant role in specifying responses to NRG stimulation in these experiments. While endogenous

ErbB3 expression permits NRG stimulation of exogenous neu tyrosine phosphorylation, NRG does not stimulate receptor tyrosine phosphorylation in cells that express exogenous EGFR only. Perhaps the level of endogenous ErbB3 expression in Ba/F3 cells is insufficient to permit NRG-induced EGFR tyrosine phosphorylation. Alternatively, while NRG induces high levels of ErbB2-ErbB3 heterodimerization and tyrosine phosphorylation, NRG induces only limited amounts of EGFR-ErbB3 heterodimerization and signaling [Pinkas-Kramarski, *et al.*, 1996; Tzahar, *et al.*, 1996; Graus-Porta, *et al.*, 1997]. Finally, intrinsic differences between the exogenous human and endogenous mouse proteins may result in the differing capacities to undergo NRG-induced heterotypic receptor interactions.

While NRG can stimulate the tyrosine phosphorylation of each receptor under the appropriate conditions, the diversity of biological responses to NRG indicates that there must be additional mechanisms by which biological responses to NRG are specified. The patterns of NRG-induced stimulation of ErbB receptor tyrosine phosphorylation and IL-3 independent survival or proliferation demonstrates that there are several hierarchical levels at which biological responses to NRG are apparently specified.

First, responsiveness to NRG requires the expression of ErbB3 or ErbB4. Previous work and results presented here establish that NRG can not bind or stimulate tyrosine phosphorylation of erbB family receptors in the absence of ErbB3 or ErbB4 expression. Biological responses to NRG are also specified by the intrinsic kinase activity of the ErbB family receptor(s) stimulated by NRG, since kinase-deficient ErbB3 requires the presence of a co-receptor for hormone-regulated phosphorylation.

Distinct biological responses to NRG are also conferred by ligand-induced coupling of different ErbB family receptors to different signaling pathways. NRG

enables the IL-3 independent survival of cell lines expressing ErbB2, probably through NRG-induced activation of ErbB2 via ErbB3. However, NRG does not enable the IL-3 independent survival of EGFR + ErbB-3 cells, or of ErbB4 cells, even though NRG stimulates receptor tyrosine phosphorylation in these lines. This demonstrates that ErbB2 has signaling properties distinct from those of the EGFR, ErbB3, or ErbB4, and is consistent with earlier work showing that different ErbB family receptors can activate different signaling pathways and responses [DiFiore, *et al*, 1990; Fedi, *et al.*, 1994; Kim, *et al.*, 1994; Prigent and Gullick, *et al.*, 1994; Soltoff, *et al.*, 1994; Carraway, *et al.*, 1995]. Neither NRG nor EGF induces IL-3-independent proliferation of cells that individually express ErbB4 or EGFR [Riese, *et al.*, 1995; Riese, *et al.*, 1996]. Yet, NRG stimulates IL-3-independent proliferation in the EGFR + ErbB-4 cell line. One simple explanation would be that IL-3-independent proliferation requires activation of two independent pathways, one of which is activated by the EGFR, and one by ErbB-4. An interesting alternative would be that the sites of EGFR and ErbB4 phosphorylation differ in ligand-induced EGFR/ErbB4 heterodimers than in ligand-induced receptor homodimers owing to substrate specificity of the receptor catalytic domains and steric considerations in the cross-phosphorylation reaction. This would permit recruitment of unique signaling proteins to the heterodimer, resulting in unique biological responses. A model whereby a receptor is phosphorylated on different tyrosine residues by direct agonist binding compared to transmodulation is supported by the observation that while EGF stimulates IL3 independence in EGFR + ErbB3 cells, NRG does not [Riese, *et al.*, 1995; Riese, *et al.*, 1996a]. Furthermore, EGF and NRG stimulate different biochemical and physiologic responses in fibroblasts engineered to express EGFR and ErbB3, suggesting that EGFR activated directly and in *trans* couple to different signaling pathways and biological responses [Cohen, *et al.*, 1996]. Thus, the diversity

of hormone-regulated outputs from this receptor outwork may extend beyond the simple combinatorial possibilities.

Since EGF and NRG bind to different receptors, it can be predicted that individual members of the EGF family of ligands activate different constellations of erbB family receptors, so that these different ligands will yield distinct patterns of biological responses. Evidence presented here supports this prediction. Previous reports demonstrated that BTC binds to the A431 human adenocarcinoma cell line and the MDA-MB-453 human breast carcinoma cell line, both of which overexpress the EGFR. This binding was quenched by the addition of an excess of EGF, suggesting that BTC is a ligand for the EGFR [Watanabe, *et al.*, 1994]. However, the effect of BTC on EGFR tyrosine phosphorylation and signaling and the possibility that BTC might activate other erbB family receptors were not assessed. Here we show that in Ba/F3 cells expressing only a single ectopic erbB family receptor, BTC stimulates the tyrosine phosphorylation of both the EGFR and, surprisingly, ErbB4 (Figure 1). This is consistent with the observation that radiolabeled BTC binds specifically to EGFR and ErbB4, but not to ErbB2 (Plowman, *et al.*, in preparation). Control experiments performed in parallel demonstrated that radiolabeled amphiregulin and EGF bound only to EGFR and radiolabeled NRG-β bound only to ErbB4, as previously reported. Thus, BTC exhibits activities that are distinct from those displayed by EGF, which activates the EGFR alone, and NRG-β, which activates ErbB3 and ErbB4 (Figure 1). Analogous experiments have demonstrated that HB-EGF and EPR also activate both EGFR and ErbB4 [Elenius, *et al.*, 1997; Riese, *et al.*, submitted] (Figure 1). Furthermore, in this first comprehensive analysis of ErbB family transmodulation for both BTC and EGF, we find that EGF can transmodulate ErbB4 in the EGFR + ErbB4 cell line. We also demonstrate that BTC stimulates a pattern of receptor

transmodulation that is qualitatively distinct from the patterns stimulated by EGF and NRG-β.

With one exception, BTC, EGF, and NRG-β transmodulated the tyrosine phosphorylation of all four ErbB family receptors in cell lines that express any receptor for each ligand. Thus, differences in ligand activities in the double recombinant cell lines can be predicted by differences in activities in the single recombinant cell lines. For example, NRG-β activates ErbB3, while BTC does not activate ErbB2 or ErbB3. Not surprisingly, in cells expressing ErbB2 + ErbB3, NRG-β stimulates the phosphorylation of both receptors, while BTC does not stimulate the phosphorylation of either receptor in this cell line (Table 1). The single exception is the response of the ErbB3 + ErbB4 (3+4) cell line to betacellulin. Both betacellulin and NRG-β stimulate ErbB4 tyrosine phosphorylation in the single recombinant cell line. However, in 3+4 cells, NRG-β stimulates the tyrosine phosphorylation of both receptors, while betacellulin stimulates the tyrosine phosphorylation of ErbB4 but not of ErbB3. Nonetheless, because NRG-β binds ErbB3, it is not clear that this absence of ErbB3 tyrosine phosphorylation is due to differences between BTC- or NRG-β-induced ErbB3 transmodulation.

Previous work demonstrated that different ErbB family receptors or combinations of receptors couple to distinct cellular signaling pathways. For example, EGFR activation stimulates the tyrosine phosphorylation of four proteins that are not highly phosphorylated following ErbB2 activation [Fazioli, *et al.*, 1992]. Furthermore, activated ErbB- stimulated higher levels of phosphatidylinositol 3-kinase than EGFR did [Fedi, *et al.*, 1994; Soltoff, *et al.*, 1994; Carraway, *et al.*, 1995], and it has been suggested that EGFR and ErbB2 bind the adapter protein GRB2, but ErbB3 does not [Prigent and Gullick, 1994; but also see Kim, *et al.*, 1994; Fedi, *et al.*, 1994]. The different coupling capacities of the erbB family receptors can be correlated

to specific biological responses. Activation of the EGFR stimulates the IL-3 independent proliferation of 32D myeloid cells, while wild-type and mutationally-activated ErbB2 alleles do not [DiFiore, *et al.*, 1990]. In Ba/F3 cells, activation of EGFR or ErbB2, but not ErbB4, stimulates IL-3 independent survival. Moreover, activation of EGFR and ErbB4 together stimulates IL-3 independent proliferation [Riese, *et al.*, 1995; Riese, *et al.*, 1996a].

We found that BTC stimulates IL-3 independent survival or proliferation in Neu + ErbB-4 cells and in every cell line that expresses EGFR. In contrast, EGF stimulated IL-3 independence only in those cell lines that express EGFR while NRG-β stimulated IL-3 independence only in the EGFR + ErbB-4 cell line and in those cell lines that express ErbB2 [Riese, *et al.*, 1995; Riese, *et al.*, 1996a]. Therefore, with a single exception, the minimal requirement for IL-3 independence is activation of either EGFR or ErbB2. The exception is that BTC and EGF, but not NRG-β, stimulated IL-3 independent survival in the EGFR + ErbB3 cell line (Table 1). This lack of response to NRG-β may merely reflect the lower level of EGFR phosphorylation stimulated by NRG-β in this cell line [Riese, *et al.*, 1995]. On the other hand, the absence of biological response may reflect the different mechanism by which the EGFR is activated. As we have discussed earlier, the mechanism of receptor activation may partially define the sites of receptor autophosphorylation and coupling to downstream signaling proteins and physiologic responses [Cohen, *et al.*, 1996; Graus-Porta, *et al.*, 1997]. We demonstrated previously that coupling of these multiple receptor species to cellular signaling pathways acts in a non-additive manner in specifying biological responses [Riese, *et al.*, 1996]. Accordingly, while activation of EGFR or ErbB2 by themselves stimulated IL-3 independent survival, activation of EGFR and either ErbB2 or ErbB4 together stimulated IL-3 independent proliferation (Table 1).

As we have discussed previously, biological responses to EGF family ligands are regulated by several hierarchical mechanisms. Some, but not all, of these mechanisms are shared by other networks of receptor tyrosine kinases and their ligands, including the neurotrophin network and the fibroblast growth factor (FGF) network. Like the EGF family, the neurotrophin and FGF ligand families have several members that can each activate multiple receptors. The neurotrophin ligand family includes nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4, also known as NT-4/5 or NT-5 [Reviewed in Barbacid, 1994], while the FGF family has at least 9 members encoded by different genes [Reviewed in Johnson and Williams, 1994]. Furthermore, like the erbB receptor family, both the FGF receptor and neurotrophin receptor families have multiple members (FGFR-1, FGFR-1, FGFR-3, FGFR-4 and TrkA, TrkB, TrkC, respectively). Moreover, like the EGFR and ErbB4, some of these FGFRs and Trks can bind multiple ligands [Reviewed in Johnson and Williams, 1994; Barbacid, 1994].

Another regulatory mechanism common to the EGF/ErbB and FGF signaling networks is that both use heparan sulfate proteoglycans (HSPGs) to modulate receptor-ligand interactions. FGFs bind with low affinity in a multivalent manner to HSPGs, causing ligand oligomerization [Reviewed in Lemmon and Schlessinger, 1994] and increasing their binding affinity for FGFRs [Reviewed in Eckenstein, 1994]. Because the FGF/FGFR complex exists in a 1:1 stoichiometry [Spivak-Kroizman, *et al.*, 1994], yet FGFs are monomeric, it has been proposed that HSPG binding potentiates FGF stimulation of FGFR phosphorylation and dimerization. HSPGs also regulate the interactions of EGF family ligands with their receptors. Several EGF family ligands bind HSPGs, including NRGs, AR, and HB-EGF, and this binding regulates ligand-receptor interactions [Aviezer and Yayon, 1994; Johnson and Wong,

1994; Cook, *et al.*, 1995a; Cook, *et al.*, 1995b]. However, many of the mechanistic details of regulation by HSPGs have yet to be elucidated.

While the neurotrophin and FGF networks have regulatory mechanisms that are also features of the EGF/erbB network, there are also features of the neurotrophin and FGF networks that are not part of the EGF/ErbB network. Alternative splicing produces truncated FGFR and Trk isoforms lacking the cytoplasmic tyrosine kinase domain and sites for tyrosine phosphorylation [Reviewed in Johnson and Williams, 1994; Barbacid, 1994. Therefore, a regulatory mechanism not observed in the EGF/ErbB network results in dominant negative receptors, which are not a characteristic of the EGF/ErbB network. Another feature that is characteristic of the neurotrophin network and not seen in the EGF/erbB network is regulation by a low-affinity co-receptor. p75, the low-affinity neurotrophin receptor, has no tyrosine kinase domain [Reviewed in Chao, 1994] and p75 binding is in some cases dispensable for biological response [Reviewed in Ibanez, 1994]. Nonetheless, it has been proposed that p75 regulates the biological response to neurotrophins by altering the affinity of neurotrophin binding to the Trk family receptors [Benedetti, *et al.*, 1993; Reviewed in Chao, 1994].

Data presented here suggests that differences in NRG-β, EGF, and BTC activities play a significant role in specifying the proliferation and differentiation of human tissues *in vivo*. These ligands may play their most significant role in the mammary epithelium and tissues of neuroectodermal origin. Not only has the expression of EGF family ligands been documented in these cell types, but these ligands can regulate the proliferation and/or differentiation of these cell types in cultured cell or animal model systems. Furthermore, mounting evidence suggests that, depending on the identity of the receptors, increased expression and/or signaling of ErbB family receptors may either stimulate or inhibit epithelial cell

proliferation and tumorigenesis. [Reviewed in Hynes and Stern, 1994; Bacus, *et al.*, 1996]. Because different ErbB family receptors stimulate different physiological responses through differential coupling to cellular signaling proteins, a major focus of our current efforts has been to identify the components of these signaling pathways. These experiments may identify genes suitable for anti-tumor gene therapy or proteins that are targets for anti-tumor small molecule inhibitors.

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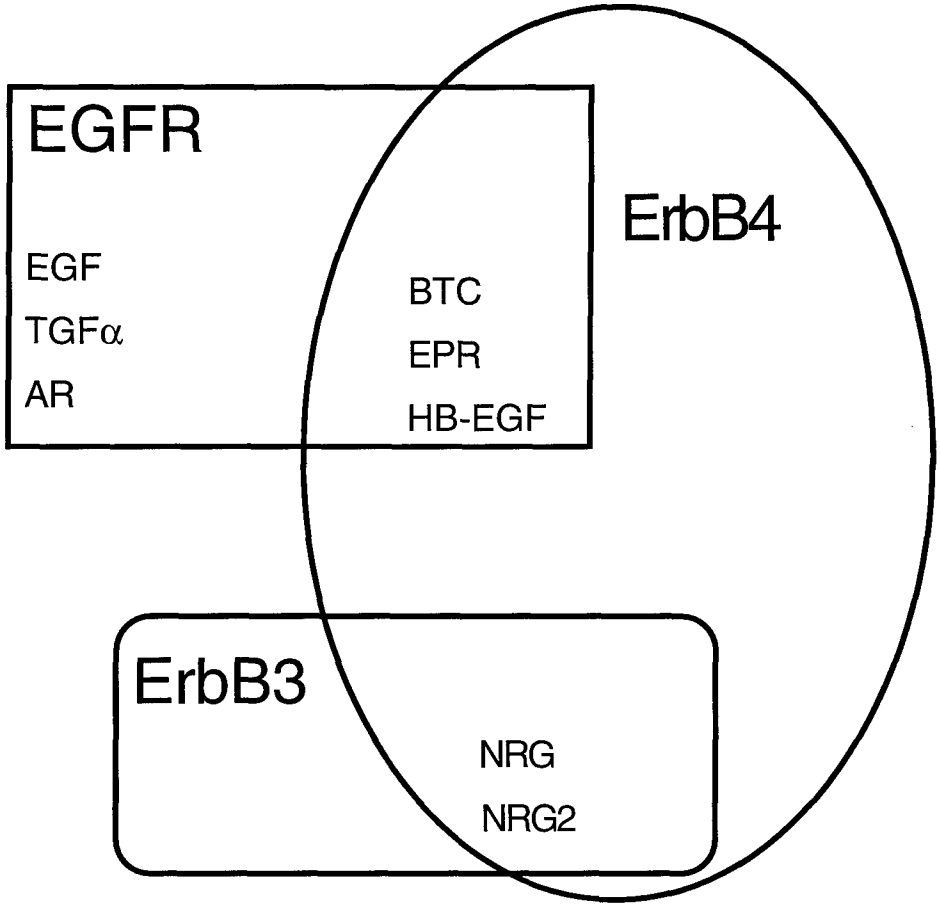
VI. Appendices

A. Figures and Tables

Figure 1

Venn Diagram Illustrating Patterns of Hormone-induced ErbB Family Receptor Phosphorylation



Data adapted from Riese, *et al.*, 1995; Riese, *et al.*, 1996a; Riese, *et al.*, 1996b; Chang, *et al.*, 1997; Riese and Stern, in press; Riese, *et al.*, submitted.

Table 1

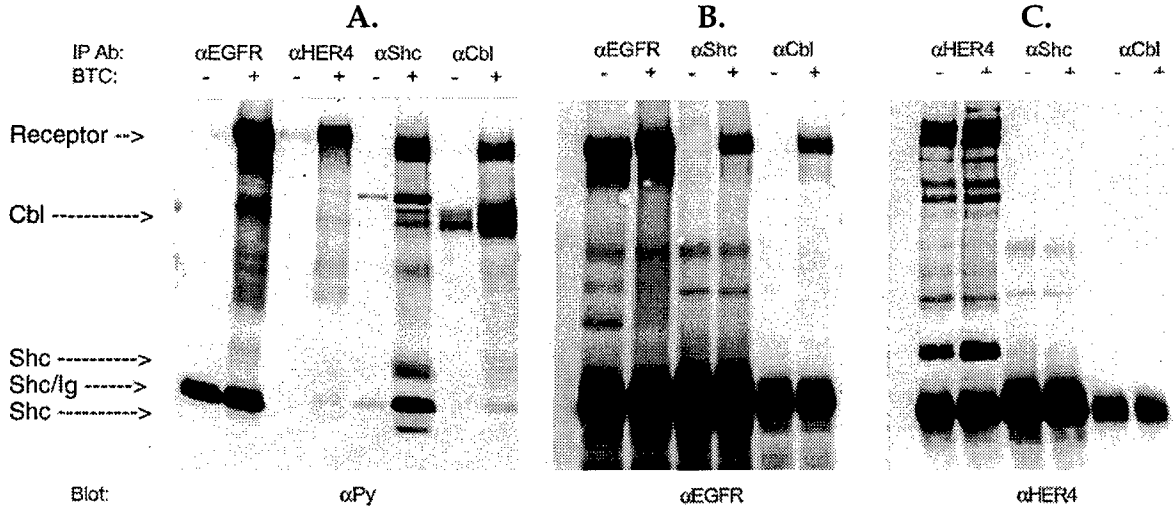
Physiologic Responses to ErbB Family Receptor Activation

Receptor(s)	Response
EGFR	IL-3-Independent Survival
Neu	IL-3-Independent Survival
ErbB-4	No Response
EGFR + Neu	IL-3-Independent Proliferation
EGFR + ErbB-4	IL-3-Independent Proliferation
Neu + ErbB-4	IL-3-Independent Survival

Data adapted from Riese, *et al.*, 1995; Riese, *et al.*, 1996a; Riese, *et al.*, 1996b.

Figure 2

Immunoprecipitation and Immunoblotting of Lysates From EGFR+ErbB4 Cells Stimulated with Betacellulin



From Riese and Stern, unpublished data.

Table 2

Reduced Drug-Resistant Colony Formation in MCF-10A Mammary Epithelial Cells Infected with the ErbB4-Neo^R Retrovirus

	Drug-Resistant Colonies	
Cell Line	**LXSN-Neu**	**LXSN-ErbB4**
Ψ2	100	18
MCF-10A	100	2

(Totals represent the normalized average of three trials)

From Riese and Stern, unpublished data.

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C. Personnel

David J. Riese II, Ph.D., Principal Investigator
David F. Stern, Ph.D., Supervisor

D. Graduate degrees resulting from contract support:
None